

Ultrastructural Localization of α -Actinin and Filamin in Cultured Cells with the Immunogold Staining (IGS) Method

GABRIELE LANGANGER, JAN DE MEY, MARC MOEREMANS, GUY DANEELS, MARC DE BRABANDER, and J. VICTOR SMALL*

Laboratory of Biochemical Cytology, Division of Cellular Biology and Chemotherapy, Department of Life Sciences, Janssen Pharmaceutica Research Laboratories, B-2340 Belgium, and *Department of Physics, Institute of Molecular Biology, Austrian Academy of Sciences, A-5020 Salzburg, Austria

ABSTRACT Monospecific antibodies to chicken gizzard actin, α -actinin, and filamin have been used to localize these proteins at the ultrastructural level: secondary cultures of 14-d-old chicken embryo lung epithelial cells and chicken heart fibroblasts were briefly lysed with either a 0.5% Triton X-100/0.25% glutaraldehyde mixture, or 0.1% Triton X-100, fixed with 0.5% glutaraldehyde, and further permeabilized with 0.5% Triton X-100, to allow penetration of the gold-conjugated antibodies. After immunogold staining (De Mey, J., M. Moeremans, G. Geuens, R. Nuydens, and M. De Brabander, 1981, *Cell Biol. Int. Rep.* 5:889–899), the cells were postfixed in glutaraldehyde-tannic acid and further processed for embedding and thin sectioning.

This approach enabled us to document the distribution of α -actinin and filamin either on the delicate cortical networks of the cell periphery or in the densely bundled stress fibers and polygonal nets. By using antiactin immunogold staining as a control, we were able to demonstrate the applicability of the method to the microfilament system: the label was distributed homogeneously over all areas containing recognizable microfilaments, except within very thick stress fibers, where the marker did not penetrate completely. Although α -actinin specific staining was homogeneously localized along loosely-organized microfilaments, it was concentrated in the dense bodies of stress fibers. The antifilamin-specific staining showed a typically spotty or patchy pattern associated with the fine cortical networks and stress fibers. This pattern occurred along all actin filaments, including the dense bodies also marked by anti- α -actinin antibodies.

The results confirm and extend the data from light microscopic investigations and provide more information on the structural basis of the microfilament system.

The motile processes of cultured cells, such as the formation of cellular contacts, cell spreading, shape changes, cell retraction, and locomotion, ruffling, surface motility, and cytokinesis, are to a large extent mediated by F-actin microfilaments (for reviews see references 4, 19, 26, 48, 61). The microfilament system in cultured cells occurs in a few characteristic patterns in the different cell domains (4, 5, 18, 28, 46): (a) In the leading edge—a highly organized three-dimensional network adjacent to parallel arrays of microspikes and filopodia; (b) in the cell body—(i) networks or mats of microfilaments differing in degree of organization and density, mainly confined to the cell cortex and (ii) prominent and densely packed microfilament bundles or “stress fibers,” their termini, and polygonal nets.

By the use of light microscopic antibody localizations (reviewed in reference 25) and microinjection studies, it has become apparent that a number of mechanochemical proteins, including α -actinin (13, 23, 38), filamin (54), tropomyosin (36, 37, 59), myosin (14, 56, 65), fimbrin (3), and vinculin (15), are associated with this actin matrix. Moreover, their characteristic distribution patterns observed at this level of resolution indicate the significant regulatory roles that these proteins play in the dynamic activities and structural support of the microfilament system.

For a better understanding of these functions it is essential to supplement the light microscopic findings with detailed information on the ultrastructural distribution of the contractile proteins.

Hitherto, immunoelectron microscopic data on cultured cells are sparse; the available reports deal with actin (58, 63), α -actinin (8, 43, 44), vinculin (8), and myosin (12, 27, 62, 66). It will therefore be necessary to determine with greater precision than heretofore the fine structural details of the filamentous elements as these occur in cultured cells.

In this study, we examined the ultrastructural distribution of α -actinin and filamin in bundled actin arrays and cortical networks of cultured cells. Although no data so far have been presented for filamin, α -actinin has been localized in stress fibers by Sanger et al. (43), with the peroxidase-antiperoxidase method. Whereas the latter investigators as well as others (12, 27, 44, 62, 63, 66) have focused their attention on the interpretation of the antibody-marker distribution, the ultrastructural preservation of microfilaments has received only passing attention. By using the immunogold staining method (11) in combination with appropriate fixation-permeabilization conditions, we have been able to allow antibody penetration and to obtain the adequate preservation of cortical networks and ultrastructural details of stress fibers such as "electron-lucid" and "electron-dense regions" (17) or "dense bodies" (61). The details of the procedure as adapted to the localization of actin-binding proteins in different regions of the cell are described in this paper. Our results bring new information on the composition of the electron-dense and lucid bands of stress fibers and on the fine distribution of α -actinin and filamin in microfilament networks of the cell cortex. The data further support the role of α -actinin and filamin in cross-linking and organizing microfilament networks and bundles in situ. This work has been presented in a preliminary form of abstracts (34, 35).

MATERIALS AND METHODS

Cell Culture

Chicken epithelial cells were isolated from 14-d-old chicken embryo lungs. The tissue was chopped with a scalpel and trypsinized in 0.25% trypsin in Ca^{++} - and Mg^{++} -free Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY), at 37°C for 20 min. The reaction was stopped with medium containing 10% fetal calf serum. After centrifugation and resuspension, the cells were transferred to a 75-cm² Falcon flask (Falcon Plastics, Oxnard, CA). The fibroblast contaminants were allowed to attach for 2 h at 37°C. The remaining cell suspension was plated in a new 75-cm² Falcon flask and allowed to attach for 12–24 h before the medium was changed.

After 3–5 d in culture the cells were briefly (2–3 min) trypsinized (with the 0.25% trypsin solution) and plated on glass coverslips for light microscopy or on Permax petri dishes (LUX, Lab-Tek Division, Miles Laboratories, Inc., Naperville, IL) for electron microscopy. After 3–5 d in culture the cells were used for immunocytochemistry.

Chicken heart fibroblasts were obtained from 14–18-d-old chicken embryos. The hearts were cut in small pieces and submitted to serial trypsinizations for 10 min at 37°C until digestion was complete. The cell suspensions were collected in culture medium containing 10% fetal calf serum, centrifuged, resuspended, and plated in 75-cm² Falcon flasks. The fibroblasts were allowed to attach for 30 min and the supernatant was discarded. After 2–3 d in culture, they were replated for immunocytochemistry as described above.

All cells were grown in Eagle's minimum essential medium (Gibco Laboratories) supplemented with 10% fetal calf serum (Gibco Laboratories). They were incubated in a humidified 5% CO_2 /air atmosphere at 37°C.

Antibody Production, Purification, and Characterization

Filamin and α -actinin were purified to homogeneity from chicken gizzard according to Small and Sobieszek (47). Chicken gizzard actin, a gift from Dr. Apolinary Sobieszek, was prepared from acetone powder essentially as described (50).

Rabbits were immunized with native proteins and the antibodies obtained were affinity purified as described (10). The specificity of the purified antibodies was tested with the newly developed immunogold-silver staining method on

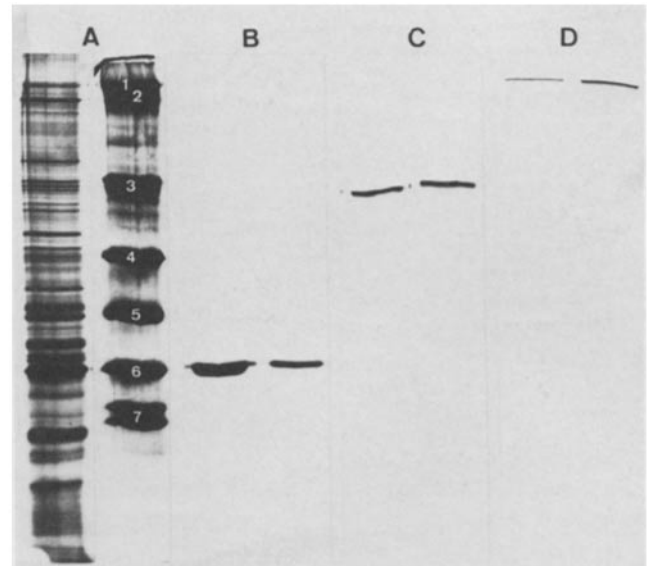


FIGURE 1 Characterization of the actin, α -actinin, and filamin antibodies by immunoblotting. Lanes in A demonstrate the electrophoretic pattern of polypeptides in SDS polyacrylamide (7.5%) gels of chicken lung epithelial cells, with total extract on the left and purified reference proteins on the right. (1) Chicken gizzard filamin; (2) chicken gizzard myosin heavy chain; (3) chicken gizzard α -actinin; (4) BSA; (5) rat brain tubulin; (6) chicken gizzard actin; and (7) pig stomach tropomyosin. Lanes in B–D illustrate the immunoblots of electroeluted proteins of chicken lung epithelial cells at the left and the purified protein mixture on the right. The single bands of B–D show the specific reactions of the antibodies (anti-actin, α -actinin, and filamin, respectively) on both the epithelial cell extract and the purified proteins.

protein blots (Moeremans, M., G. Daneels, A. Van Dijk, and De Mey, submitted for publication). Briefly, the SDS-separated proteins (32) were electrotransferred (52) onto nitro-cellulose-paper (0.45- μm). The blot units (see figure legend) were quenched with 5% BSA in 20 mM Tris-buffered saline ([TBS],¹ see reference 11) pH 8.2, at 37°C for 30 min. They were incubated with first antibodies (1 $\mu\text{g}/\text{ml}$ in TBS + 0.1% BSA, 3 ml), for 2 h and washed in TBS + 0.1% BSA. The antigen-antibody complexes were visualized by incubation for 1 h with monospecific goat anti-rabbit (GAR) IgG adsorbed to ± 20 nm colloidal gold (blotting grade), (GAR G BL, Janssen Life Sciences Products, Beerse, Belgium), diluted 1:50 to give optical density, $\text{OD}_{520} = 0.2$. The pink color produced by accumulation of gold particles at antibody-binding sites was enhanced by silver precipitation using a modification (submitted for publication) of Danscher's method (9): the blots were incubated in the dark for 5 min in Danscher's solution of silver lactate and hydroquinone (gum arabic was omitted) in citrate buffer pH 3.5. This silver enhancement results in sharply contrasted black bands corresponding to the antibody-binding sites on the blot. The staining pattern of the blot was related to the protein pattern in the acrylamide gel as revealed with silver staining (39).

Immunocytochemistry

FIXATION AND PERMEABILIZATION: For light microscopy, cells were immersed in 0.5% glutaraldehyde in PIPES buffer 1 (see reference 46: Hanks' balanced salt solution [NaCl, 137 mM; KCl, 5 mM; Na_2HPO_4 , 1.1 mM; KH_2PO_4 , 0.4 mM; NaHCO_3 , 4 mM; glucose, 5.5 mM]; PIPES, 5 mM; MgCl_2 , 2 mM; EGTA, 2 mM; pH 6.1), for 10 min followed by 0.5% Triton X-100-permeabilization, etc., as described below.

For electron microscopy of cortical networks, preparations were fixed and extracted for 2 min in a 0.5% Triton X-100/0.25% glutaraldehyde mixture (29, 45) in PIPES buffer 1. For immunoelectronmicroscopy of stress fibers, the cells

¹ Abbreviations used in this paper: GAR-FITC, goat anti-rabbit IgG-fluorescein isothiocyanate; GAR-G5, goat anti-rabbit IgG coupled to 5-nm gold; HMM-S₁, heavy meromyosin-subfragment 1; MF, microfilament; NGS, normal goat serum; TBS, Tris-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

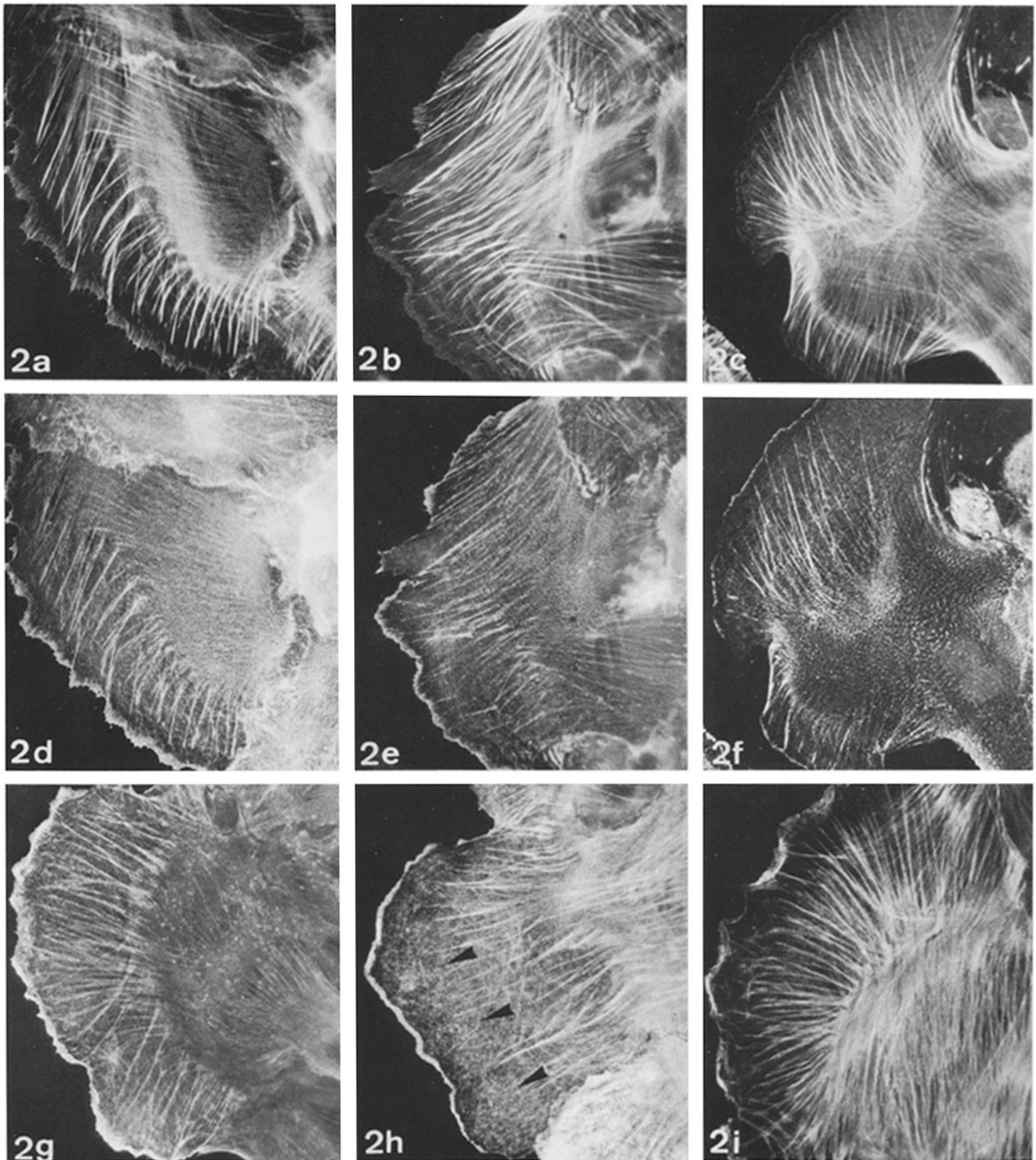


FIGURE 2 Double-fluorescence microscopic images of chicken lung epithelial cells stained with primary antibodies, GAR-FITC, and phalloidin-TRITC. (a) Directly glutaraldehyde- (0.5%) fixed cell. F-actin filaments stained with phalloidin-TRITC reveal the actin organization of a well-fixed cell: dominant stress fibers, cortical networks adjacent to fine bundles, and a bright leading edge. (b) Epithelial cell permeabilized with the 0.5% Triton X-100/0.25% glutaraldehyde mixture for 2 min and stained with phalloidin-TRITC. The general actin arrangements appear essentially unchanged. (c) 0.1% Triton X-100-treated cell for 40 s. Phalloidin-TRITC staining does not reveal any changes in stress fibers, but the beginning of microfilament extraction can be noticed at the leading edge and the cortical layer of filaments located directly behind it. (d) The α -actinin staining of the same cell as in a shows the periodic fluorescence along stress fibers and the homogeneous staining of cortical networks at the cell periphery. (e) The same cell as in b showing the characteristic and unchanged α -actinin localization at the cortical networks and microfilament bundles. (f) This picture illustrates the α -actinin staining of the 0.1% Triton X-100-extracted cell seen above. The periodicities along stress fibers appear essentially unaltered but slightly sharper in comparison with the glutaraldehyde-fixed cell. (g) 0.5% glutaraldehyde-fixed cell after antifilamin staining shows the nearly homogeneous distribution along all MF-arrays. (h) Permeabilized cell with 0.5% Triton X-100/0.25% glutaraldehyde for 2 min after staining with antifilamin. An especially large peripheral lamella shows the well-preserved cortical network in this region (arrowheads). (i) The filamin distribution in the 0.1% Triton X-100-treated cell seems unchanged in bundled MF arrays but diminished in the fine networks. $\times 560$.

were extracted with 0.1% Triton X-100 in PIPES buffer 2 (Hanks' balanced salt solution; PIPES, 5 mM; MgCl₂, 2 mM; pH 6.1, lacking EGTA): 40 s for epithelial cells and 20 s for fibroblasts. Both preparations were subsequently fixed in 0.5% glutaraldehyde in PIPES buffer 1 for 10 min. The cells were further washed three times in buffer 1; permeabilized with 0.5% Triton X-100 in PIPES buffer 1 for 30 min; washed three times in PIPES buffer 1; treated with sodium borohydride (57) 1 mg/ml in PIPES buffer 1 for 20 min; immersed in 20 mM TBS (Tris-buffered saline, pH 8.2, see reference 11), +0.1% BSA, and washed three times in TBS + 0.1% BSA.

INCUBATION WITH PRIMARY ANTIBODIES: The fixed and permeabilized cells were incubated in NGS (normal goat serum, diluted 1:20 in TBS + 0.1% BSA) for 15 min and subsequently with the first antibody in concentrations of 2–5 μg/ml in TBS + 0.1% BSA for 2 h or overnight (equivalent results were obtained). Control preparations were incubated for the same time in 1% NGS in TBS + 0.1% BSA. Cells were further washed in TBS + 0.1% BSA (three times for 10 min).

DOUBLE-LABEL IMMUNOFLUORESCENCE: The second antibody, goat anti-rabbit IgG-fluorescein isothiocyanate (GAR-FITC) (Nordic, Tilburg,

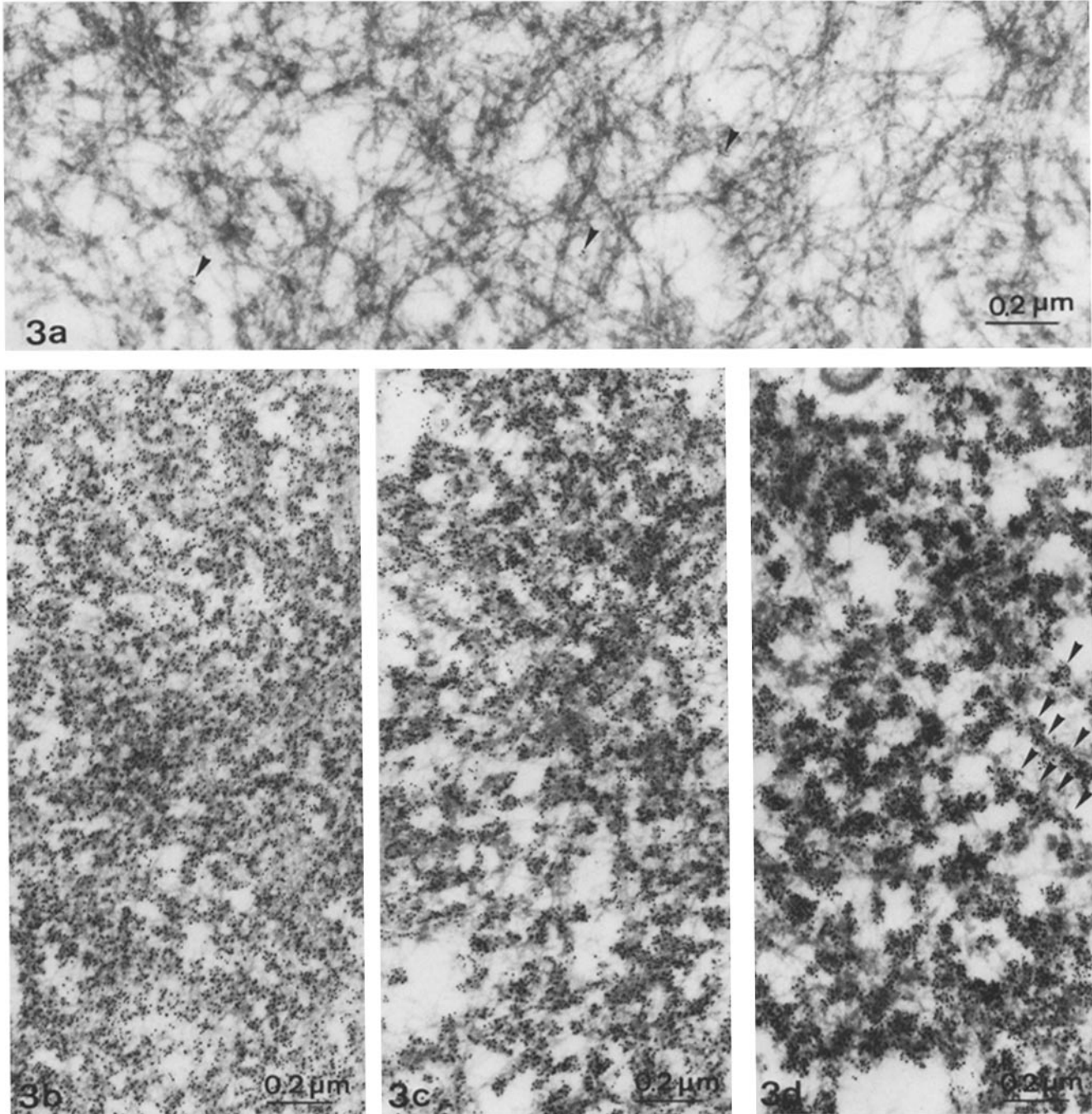


FIGURE 3 Thin sections of cortical networks of epithelial cells fixed and permeabilized with 0.5% Triton X-100/0.25% glutaraldehyde for 2 min. (a) 1% NGS control. The nonspecific background staining is extremely low (arrowheads). The preservation of the cortical filaments after the mix-fixation procedure, embedding, and thin sectioning is illustrated in this figure. (b) Antiactin control. Homogeneously dispersed label along MFs demonstrates how the filamentous network is stained by gold-labeled antibodies. (c) The α -actinin-specific marker shows no noteworthy difference from antiactin staining. MFs arranged in a network are covered by a mat of gold particles. (d) Antifilamin-specific staining shows a patchy arrangement of marker groups along actin filaments (arrowheads). Meshworks of actin are densely covered by these groups. There is no indication at this level of resolution that special sites of this network (e.g., cross points) are preferred by filamin. (a, c, and d) $\times 53,650$; (b) $\times 53,520$.

The Netherlands) diluted 1:40 in TBS + 0.1% BSA and mixed with 10^{-8} M rhodamine-labeled phalloidin (64) (generously donated by Professor T. Wieland, Heidelberg, Federal Republic of Germany), was applied to the cells for 1 h. The preparations were washed three times for 10 min in TBS + 0.1% BSA, rinsed in water, and mounted in Gelvatol supplemented with 100 mg/ml 1,4-diazobicyclo-[2.2.2]-octane (33). The cells were viewed with a Reichert Polyvar microscope (Reichert Vienna, Austria) equipped with epifluorescence optics, and photographed with a Kodak Tri-X-Pan film (Eastman Kodak Co., Rochester, NY).

ELECTRON MICROSCOPY: The secondary antibody (goat anti-rabbit IgG) coupled to 5-nm gold (GAR-G5) (Janssen Life Sciences Products) was used either undiluted for 4 h (optical density at 520 nm $[OD_{520}] = 5$) or diluted 1:4 ($OD_{520} = 1$) in TBS + 0.1% BSA overnight (equivalent results were obtained). The cells were washed in TBS + 0.1% BSA, three times for 10 min, then washed twice in Sørensen's phosphate buffer, pH 7.2 (P_i buffer: 36 ml of 0.2 M Na_2HPO_4 + 14 ml 0.2 M NaH_2PO_4 diluted to 100 ml with H_2O); postfixed in 1% glutaraldehyde + 0.2% tannic acid in P_i buffer, for 30–60 min, washed thoroughly in P_i buffer, postfixed in 0.5% osmium tetroxide (OsO_4) in P_i buffer, 10 min on ice; washed in P_i buffer, three times; partially dehydrated in 70% ethanol (several changes); impregnated with 0.5% uranyl acetate + 1% phosphotungstic acid in 70% ethanol for 30 min, and further dehydrated in alcohol and embedded in Epon. The cells were selected by phase-contrast microscopy, marked with a diamond pencil, reembedded with the cell side up, thin-sectioned, and viewed with a Philips EM 410 microscope.

Heavy Meromyosin-Subfragment 1 (HMM-S₁) Decoration

HMM-S₁ (generously donated by Dr. Apolinary Sobieszek, Salzburg, Austria) was applied in concentrations of 0.5–1 mg/ml in PIPES buffer 2 to unfixed

cytoskeletons prepared as described and allowed to react for 10 min. After brief washing in PIPES buffer 2, the cells were fixed in 0.5% glutaraldehyde and treated for electron microscopic α -actinin localization as above. All experiments were carried out at room temperature unless otherwise stated.

RESULTS

Characterization of Antibodies

The immunoblots revealed that antiactin, anti- α -actinin, and antifilamin reacted with bands having the same relative mobility as purified actin, α -actinin, and filamin in both purified reference proteins and epithelial cell total extracts (Fig. 1). The single bands in each blot unit demonstrate that the antibodies are monospecific against these proteins.

Light Microscopy

Double immunofluorescence has been used to document possible changes in actin and associated-protein distribution with increasing strength of permeabilization conditions. The images of directly 0.5% glutaraldehyde-fixed preparations (Fig. 2, *a, d, and g*), which are consistent with other data from the literature, were used as reference for any deviations caused by different extraction media:

(*a*) The general actin arrangements of a 0.5% glutaraldehyde-fixed epithelial cell were revealed by phalloidin tetramethyl-

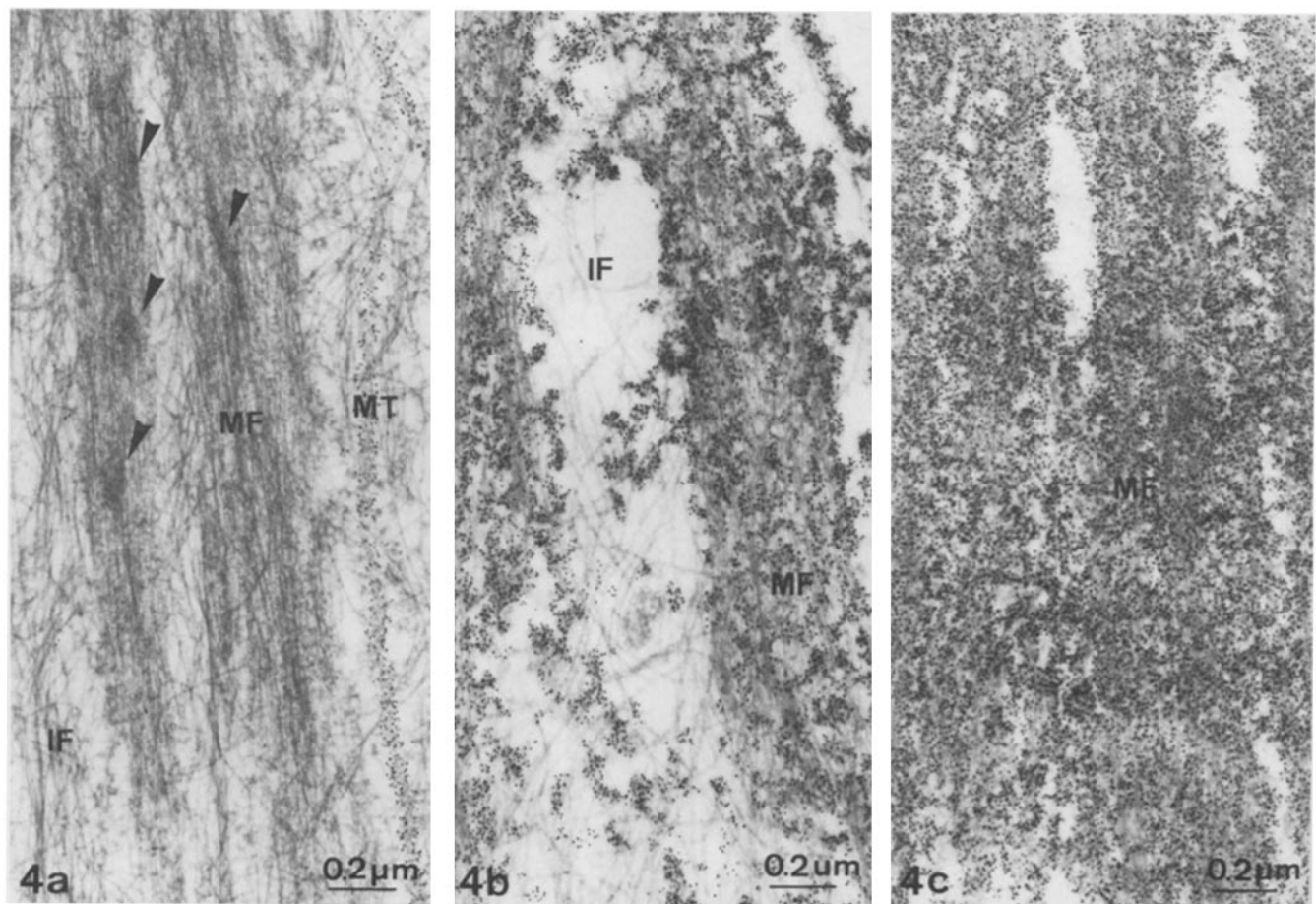


FIGURE 4 Controls for method specificity on 0.1% Triton X-100-treated epithelial cells (40 s) and fibroblasts (20 s). (*a*) The antitubulin- and GAR-G5-stained epithelial cell demonstrates the preservation of the dense bodies of stress fibers after the labeling procedure (arrowheads) and the general morphology of microfilaments (MF). Only microtubules (MT) are decorated with gold particles. Intermediate filaments (IF) and microfilaments (MF) are unstained. (*b*) Antiactin- and GAR-G5-stained epithelial cell. Intermediate filaments (IF) are unmarked. (*c*) Antiactin- and GAR-G5-stained fibroblast. Both *b* and *c* demonstrate the penetration of gold-labeled antibodies into the densely bundled stress fibers (MF). The observed background in both preparations is negligible. $\times 45,600$.

rhodamine isothiocyanate (TRITC) staining. As shown previously (64) fluorescent phalloidin can bind to glutaraldehyde-fixed cytoskeletons and serve for the visualization of cellular F-actin. α -Actinin staining was arranged in an alternating periodic pattern along stress fibers and was concentrated at foci of polygonal nets and cell-cell contacts. The leading edge, cortical networks, and occasionally fine long rays of stress fibers were homogeneously stained by α -actinin (Fig. 2*d*; references 23, 24, 38, 41). Filamin showed an even distribution along all actin filaments (Fig. 2*g*; references 24, 41, 54). At higher magnifications we occasionally observed a patchy staining pattern at fine networks and periodic regions of somewhat increased fluorescence along stress fibers (see also reference 41).

(b) The Triton X-100-glutaraldehyde mix procedure had no obvious effect on the order of actin filaments as seen after phalloidin-TRITC staining (Fig. 2*b*). Generally α -actinin (Fig. 2*e*) and filamin (Fig. 2*h*) exhibited the same distribution pattern as seen in glutaraldehyde-fixed preparations.

(c) Effects of brief 0.1% Triton X-100 treatment can be summarized as follows: the number of fine cortical filaments directly behind the leading edge was greatly reduced as demonstrated by the phalloidin-TRITC staining in Fig. 2*c* and by the antibody preparations in Fig. 2, *f* and *i*. Cortical networks

more centrally in the cell body were occasionally preserved (Fig. 2*c* and *f*) or partially extracted (Fig. 2*i*).

The microfilament bundles of stress fibers and polygonal nets, visualized by phalloidin-TRITC (Fig. 2*c*), seemed unaltered.

The antibody-staining patterns of associated proteins on stress fibers or polygonal nets also remained essentially unchanged. However, the periodic α -actinin fluorescence appeared sharper and brighter (Fig. 2*f*). This could be due to some loss of α -actinin, first noticed at regions of low α -actinin concentration as in between the highly fluorescent bands. In addition, the possibly easier access of antibodies into highly cross-linked structures could result in a sharper and more contrasted staining pattern.

Differences in filamin distribution along stress fibers after Triton X-100 extraction could not be observed at the light microscopic level (Fig. 2*i*).

Electron Microscopy

CORTICAL NETWORKS: The loose networks beneath the plasma membrane are known to be very susceptible to the fixation procedure involved. In light microscopic preparations of 0.5% glutaraldehyde and Triton X-100-glutaraldehyde

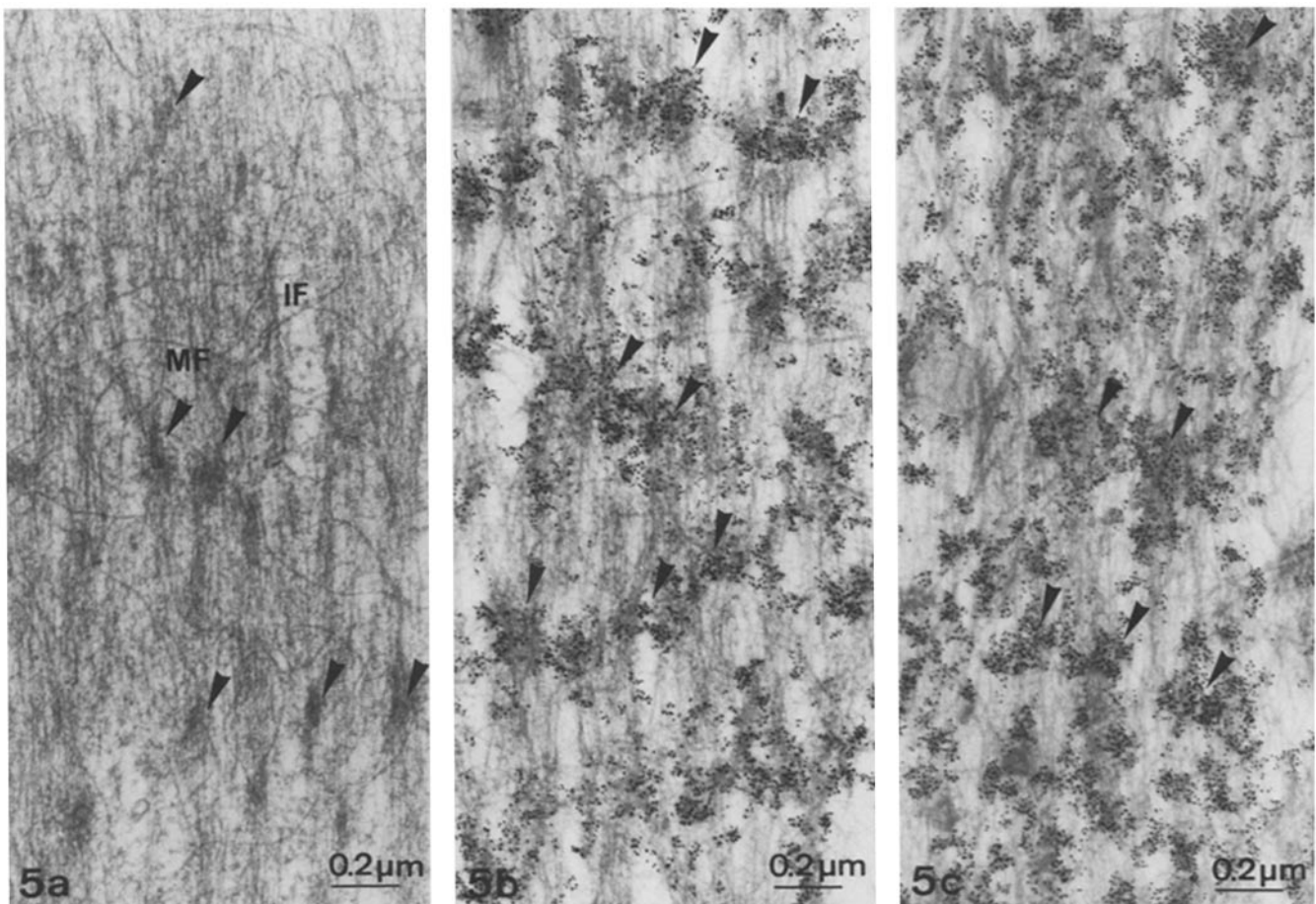


FIGURE 5 Comparison of anti- α -actinin- and antifilamin GAR-G5-stained chicken lung epithelial cells after Triton X-100 extraction (0.1%, 40 s) with the conventional morphology of glutaraldehyde-fixed cells. (a) The 2.5% glutaraldehyde-fixed cell shows the microfilament bundles (MF) containing dense bodies (arrowheads). Epithelial cells are particularly rich in intermediate filaments (IF). (b) The α -actinin label is concentrated in dense bodies still visible after immunolabeling. Their shape and distance correlate to those in a (arrowheads). The intervening electron-light zones are much less decorated. (c) Filamin staining alone MF bundles. The arrowheads indicate the concentrated stain in dense bodies. The label in the electron-light zones is clearly visible. The patchy pattern both in dense and light zones resembles the one observed in cortical meshworks. $\times 45,650$.

mix-fixed epithelial cells, the cortical networks were especially obvious at the cell periphery (see arrowheads in Fig. 2*h*) but also present in between stress fibers in close vicinity to the membrane (see Fig. 2, *b*, *e*, and *h*). The adequate preservation of a corresponding peripheral network after Triton X-100-glutaraldehyde mix treatment, incubation with 1% NGS, immunogold staining, postfixation, and thin sectioning is shown in Fig. 3*a*. The unspecific background revealed in the same micrograph (arrowheads, Fig. 3*a*) is negligible. The mix procedure also provided optimal conditions for antibody labeling, as illustrated in Fig. 3, *b*, *c*, and *d*. The antiactin reference (Fig. 3*b*) showed a homogeneous labeling of the microfilament network. Due to the high density of gold particles, individual filaments could only be recognized over very short distances.

α -Actinin-specific label (Fig. 3*c*) decorated the networks in the form of small patches but did not differ significantly from the actin pattern (Fig. 3*b*). Antifilamin staining appeared in groups or patches of gold particles aligned along actin filaments (arrowheads in Fig. 3*c*). Networks of microfilaments were densely covered by the filamin-specific patches. Essentially the same results were obtained with fibroblasts (observations not shown).

MICROFILAMENT BUNDLES: Although densely bundled actin arrays were not sufficiently penetrated by the gold probe after the mix-fixation procedure, brief lysis in 0.1% Triton X-100 in PIPES buffer 2 (before fixation) provided the appropriate labeling conditions for these structures.

Controls were performed with 1% NGS in TBS + 0.1% BSA and antitubulin (only the antitubulin control is shown in Fig. 4*a*). No significant background staining occurred in the control preparations. Antitubulin (Fig. 4*a*) marked the microtubules without labeling microfilaments or intermediate filaments. The ultrastructure of stress fibers after immunogold staining can be evaluated in the same micrograph: the typical electron-dense and lucid bands (17) are still visible (arrowheads, Fig. 4*a*). Comparison with conventional glutaraldehyde-fixed preparations (Figs. 5*a* and 6*a*) indicates that the general appearance of stress fibers has not been altered by the procedure.

To demonstrate the penetration of the marker into the very densely packed stress fibers, it was necessary to repeat the actin control (Fig. 4, *b* and *c*). Although large portions of stress fibers were reproducibly labeled with antiactin, anti- α -actinin, and antifilamin, some very thick stress fibers showed no marker at their center (not shown). Observations using

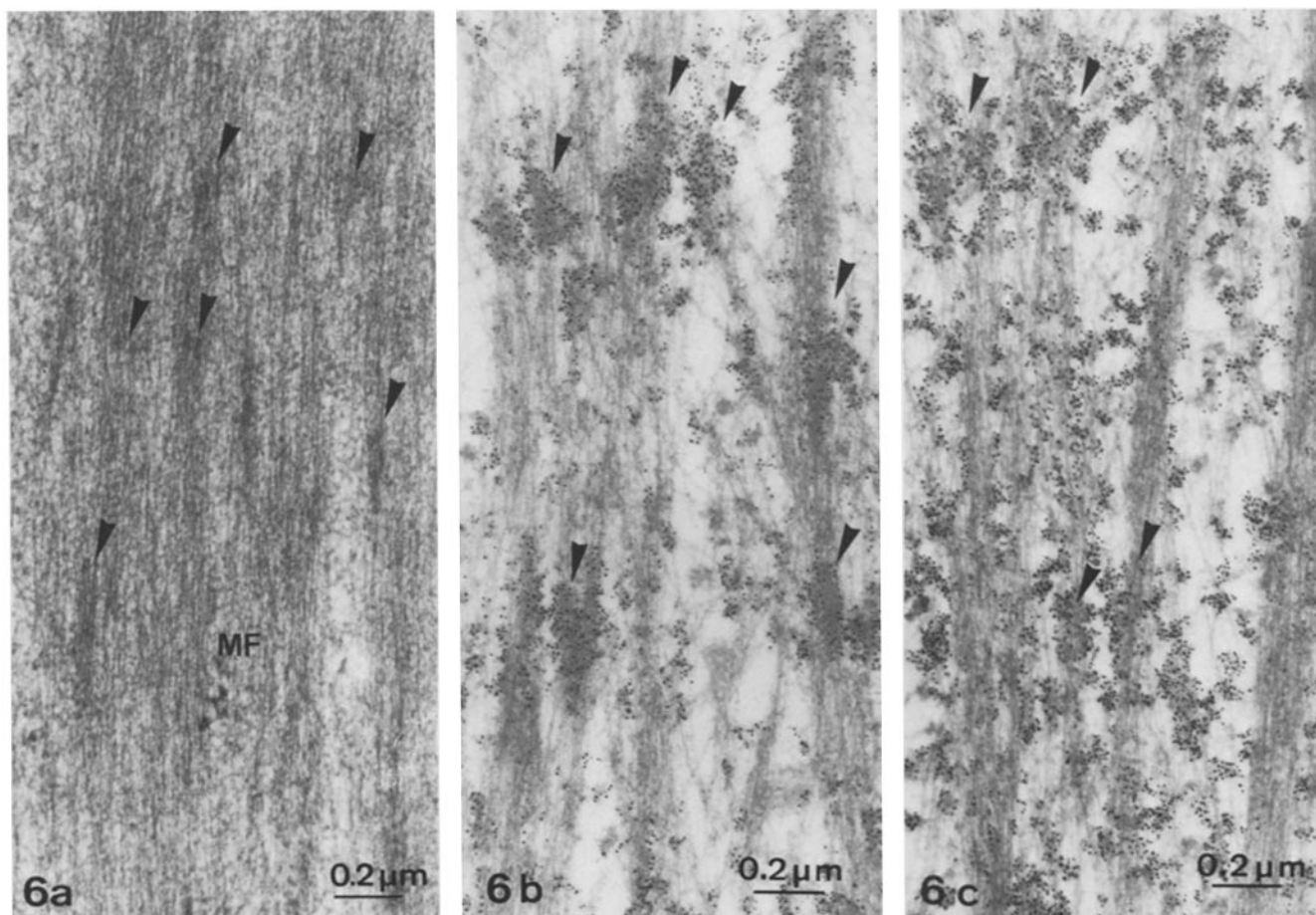


FIGURE 6 Anti- α -actinin and antifilamin staining of Triton X-100- (0.1%, 20 s) treated fibroblasts compared with the conventional GA morphology. (a) The glutaraldehyde (2.5%) morphology of stress fibers (MF) resembles that of epithelial cells. However, dense bodies (arrowheads) are less contrasted than those of epithelial cells. (b) Anti- α -actinin staining of stress fibers. Dense bodies (arrowheads) are heavily labeled while the intervening zones show only few gold particles. In comparison with epithelial cells, fibroblast-dense bodies are often thinner and longer in shape although in this respect great variations occur in both cell types. (c) Antifilamin staining in a comparable stress fiber region. Dense bodies are indicated with arrowheads. Here, too, the patchy filamin stain can be observed in electron-light and -dense zones. $\times 45,600$.

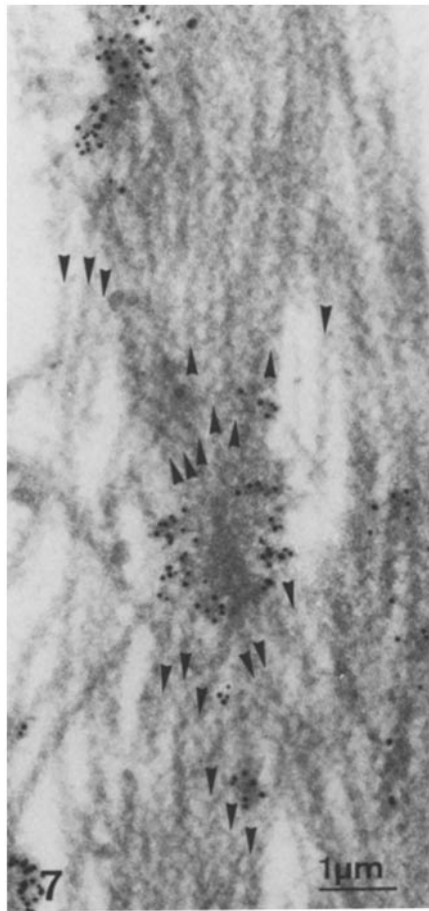


FIGURE 7 Fibroblast treated with 0.1% Triton X-100 for 20 s, decorated with HMM-S₁ for 10 min, fixed (0.5% glutaraldehyde, 10 min), and stained with anti- α -actinin and GAR-G5. Arrowheads indicate actin polarity. Filaments with opposite polarity emerge from the dense body identified with the gold marker in the middle. From another α -actinin-containing structure (dense body) in the upper left of the figure, actin fibers with polarity opposite to the first one reach into the electron-light band. $\times 108,800$.

this method are therefore restricted to the completely labeled zones.

Two types of cultured cells, fibroblasts and epithelial cells, were used to examine the ultrastructural distribution of α -actinin and filamin in stress fibers. In both cell types α -actinin was concentrated in the electron-dense bands (Figs. 5*b* and 6*b*). This was predicted (23, 24, 38, 41) and shown by Sanger et al. (43) with the peroxidase-antiperoxidase method. However, ultrastructural studies with antimyosin have led to the conclusion that myosin is localized within the dense regions of stress fibers (12, 17, 27, 66). With the punctate gold particles and the preembedding procedure used in this work, we now can directly correlate the α -actinin stain to the dense bodies (Figs. 5*b* and 6*b*). The latter are well preserved and correspond to those seen in 2.5% glutaraldehyde-fixed preparations (Figs. 5*a* and 6*a*). Electron-lucid regions, in contrast, contain no or much less α -actinin.

In stress fibers of fibroblasts and epithelial cells, filamin was localized in both electron-dense and lucid bands (Figs. 5*c* and 6*c*) in patches similar to the ones observed in cortical networks. In comparison with the electron-light regions, the gold patches in the dense bands were more densely packed (arrowheads in Figs. 5*c* and 6*c*). This could be the basis for the

occasionally observed periodic appearance in fluorescence preparations, the dense bands showing a somewhat brighter fluorescence than the intervening zones.

The polarity of microfilaments relative to dense bands has been studied previously (42). The α -actinin staining of the HMM-S₁-labeled cytoskeletons made it possible to identify the dense bands more positively, than after HMM-S₁ labeling alone (2). Most of the filaments showed arrowheads pointing away from either side of the dense bands (Fig. 7). In the intervening light regions, filaments of mixed polarity were observed. In addition, the filaments often showed some splaying, with the dense bands as regions of filament convergence.

Polygonal nets described by using immunofluorescence staining (24, 37, 38, 41), thin-sectioning techniques (24, 41), and time-lapse cinematography (30), are known to contain α -actinin and filamin at their foci. After staining by the immunogold procedure, these foci, however, often contained no label in their center (Fig. 8, *a* and *b*). This was probably due to inadequate penetration of gold particles into these regions. We therefore have to restrict our interpretations to the interconnecting fiber bundles, which were heavily labeled with α -actinin in repeating units comparable with the dense bodies of stress fibers (Fig. 8*a*).

Filamin-specific staining was observed along the whole length of interconnecting fibers in its typical patchy pattern (Fig. 8*b*).

DISCUSSION

Method

The use of colloidal gold-labeled antibodies as a marker for actin-associated proteins has many advantages: in comparison with the peroxidase procedure, colloidal gold does not need any amplification reaction to detect the label. Artifacts due to diffusion of reaction products can be avoided. There is no ambiguity as to whether a structure is labeled or not. In comparison with ferritin, it is more electron dense and allows the cell structures to be contrasted using tannic acid and heavy metals as described in this paper. As a result, the ultrastructural appearance of microfilaments is maintained except in regions of extremely dense labeling where the particles themselves cover the filaments. A disadvantage of particulate markers is that they penetrate less easily into the cytoplasmic matrix. Even with the smaller particle size of 5 nm, one is forced to use strong permeabilization conditions to label bundled actin arrays.

In the foreseeable future, techniques using 1–3-nm gold directly coupled to primary antibodies in combination with microinjection may overcome these limitations and increase the resolution closer to the level of individual molecules. Until now, however, we have relied on the use of fixed and permeabilized cells and have employed a relatively large marker (5-nm gold) for immunoelectron microscopy. As shown in this report, the present method serves as a useful tool for investigating the regional distribution of antigens with respect to morphologically-defined structures at the ultrastructural level.

Cortical Networks

Microfilament networks in the vicinity of or in close association, with the cell membrane have been described by several authors (1, 4, 5, 16, 20, 48, 51, 61), although the networks have never attracted as much attention as microfilament bundles.

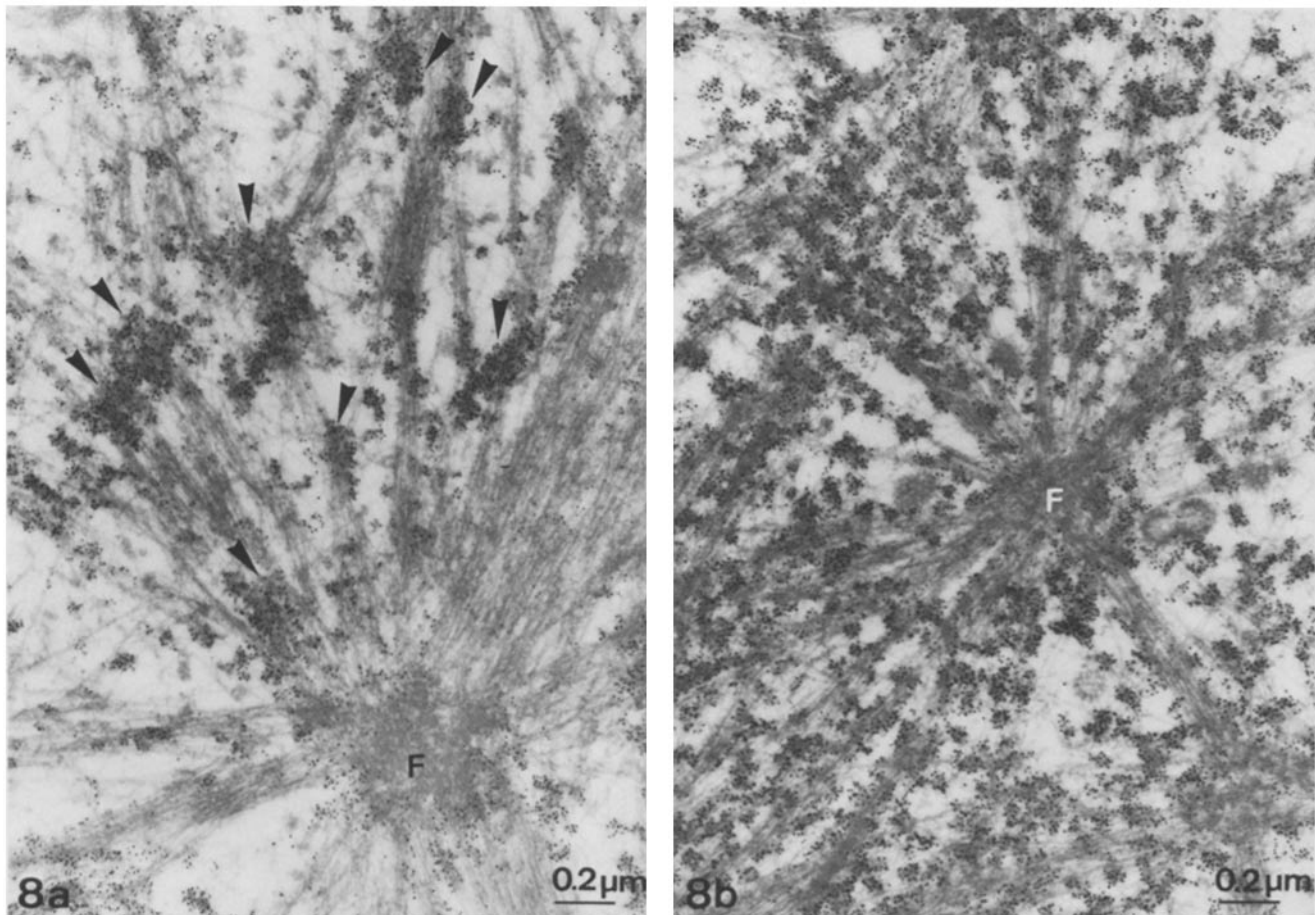


FIGURE 8 Two centers of polygonal nets with interconnecting fibers, stained with either (a) anti- α -actinin, or (b) antifilamin, both on Triton X-100- (0.1%, 20 s) treated fibroblasts. The foci in a and b (F) are marked at their surface but not at their center. Interconnecting fibers are built up of "repeating units" similar to stress fibers, best seen in the α -actinin-stained preparation (a). Dense bodies are indicated by arrowheads. Antifilamin patches are distributed almost homogeneously along fiber bundles. $\times 39,600$.

The first immunoelectron microscopic description of the cortical layer was provided by Webster et al. (58), who used a ferritin marker in conjunction with actin antibodies. No ultrastructural localization data have hitherto been presented for actin-binding proteins such as α -actinin and filamin on cortical meshworks. The adequate preservation of these actin assemblies is very susceptible to the preparative procedures employed.

Addition of glutaraldehyde in the permeabilization buffer (29, 45) provided the appropriate conditions for preserving the fragile cortical meshworks. The presence and preservation of this meshwork after the labeling procedure is documented in the NGS control. Its actin nature is revealed by the extensive labeling of the cortical filaments by actin antibodies. This network contains α -actinin in a similarly dispersed way suggesting that it is homogeneously bound along actin filaments. Filamin, in contrast, shows a sequence of distinct patches covering single filaments or bundles and networks. The patchy distribution of antifilamin staining may indicate that the binding sites of filamin along microfilaments show a more distinct spacing than α -actinin molecules. Both proteins cover large parts of the filamentous system, and are clearly associated with F-actin. From the ability of these proteins to cross-link F-actin *in vitro* (7, 31, 49, 53, 55, 60) and our own localization data, we presume that they serve to cross-connect

and stabilize loose actin assemblies like cortical networks of cultured cells.

Microfilament (MF) Bundles

Within the architecture of MF bundles or "stress fibers", electron-dense material resembling the dense bodies of smooth muscle cells and Z-lines of striated muscle has been observed (61). Tannic acid enhances the contrast of these electron-dense bands in relation to the intervening electron light or lucid bands (17). The composition of these dense bodies has been investigated by several authors. However, results obtained with myosin antibodies at the one hand (12, 27, 66) and α -actinin on the other (23, 38, 41) were contradictory. Both proteins were attributed to dense bodies whereas comparative immunofluorescence and ultrastructural studies have led to the conclusion that α -actinin and myosin are not co-localized (23, 24, 66). Only recently Sanger et al. (43), using the peroxidase-antiperoxidase procedure have demonstrated the distribution of α -actinin in stress fibers at the electron microscopic level. However, the association of α -actinin with dense bodies could only be deduced indirectly.

With the distinct localization of α -actinin with gold particles we provide direct evidence that the dense bodies are α -actinin containing structures. Moreover, we have shown that these sites also contain filamin.

Our results with HMM-S₁ arrowhead labeling correlate with those of Sanger and Sanger (42), who have proposed a model for stress fiber organization. In view of the apparent similarities with the organization of striated muscle myofibrils, they (43) as well as others (23) have called the building units of stress fibers "sarcomeric units." The implications, however, of this proposed organization of stress fibers with regard to their function in living cells, particularly concerning the question of whether or not they are contractile (see reference 6 for review), remain to be defined. We will therefore refer to stress fibers as being composed of "repeating units," rather than "sarcomeric units."

At the center of a unit—the dense body— α -actinin and filamin are accumulated, probably both acting there as filament cross-linkers. In this respect, our results clearly establish that dense bodies share common properties with the Z-bands of striated muscle, which also contain α -actinin (40) and filamin (21, 22). Further work, aimed at better understanding how stress fibers are formed, will be necessary to evaluate the extent to which these systems are analogous.

Filamin is also localized at the electron-lucid bands of stress fibers. Here it may be responsible for maintaining the parallel microfilament arrangements. By cross-linking the antiparallel microfilaments in the electron-lucid bands, filamin could also inhibit their relative sliding unless some mechanisms exist that are able to control the bonds between microfilaments and filamin. However, evidence of such mechanisms in living cells acting on stress fibers is still lacking.

The interconnecting fiber bundles of polygonal nets are built up of "repeating units" closely resembling those of stress fibers. In contrast to dense bodies, their foci are only poorly penetrated by the marker. In that these structures are well labeled by the smaller FITC-coupled antibodies, we presume that these foci are more strongly cross-linked than the dense bodies and do not allow penetration of gold particles into their center.

In conclusion, the data presented here shed new light on the differential distribution of the two actin-binding proteins α -actinin and filamin on the cytoplasmic microfilament system. Further work is aimed at better defining the functions that these and other proteins play in the dynamic organizations associated with the filamentous elements of the cytoplasmic matrix.

We are very grateful to Dr. Apolinary Sobieszek for his gift of purified chicken gizzard and HMM-S₁, and to Professor T. Wieland for his gift of TRITC-phalloidin. We thank Lambert Leijssen and Guy Jacobs for their excellent photographic work and B. Wouters for typing the manuscript.

Received for publication 7 February 1984, and in revised form 4 June 1984.

REFERENCES

- Allison, A. C., P. Davies, and S. De Petris. 1971. Role of contractile microfilaments in macrophage movement and endocytosis. *Nature (Lond.)* 232:153-155.
- Begg, D. A., R. Rodewald, and L. I. Rebhun. 1978. Visualization of actin filament polarity in thin sections. *J. Cell Biol.* 79:846-852.
- Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335-340.
- Buckley, I. K. 1981. Fine-structural and related aspects of nonmuscle-cell motility. *Cell. Muscle Motil.* 1:135-203.
- Buckley, I. K., and K. R. Porter. 1967. Cytoplasmic fibrils in living cultured cells: a light and electron microscopic study. *Protoplasma* 64:349-380.
- Burridge, K. 1981. Are stress fibres contractile? *Nature (Lond.)* 294:691-692.
- Burridge, K., and J. R. Feramisco. 1981. Non-muscle α -actinins are calcium-sensitive actin-binding proteins. *Nature (Lond.)* 294:565-567.
- Chen, W.-T., and S. J. Singer. 1982. Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J. Cell Biol.* 95:205-222.

- Danschger, G. 1981. Localization of gold in biological tissue. A photochemical method for light and electron microscopy. *Histochemistry* 71:81-88.
- De Mey, J. 1983. Raising and testing antibodies for immunocytochemistry. In *Immunocytochemistry: Practical Applications in Pathology and Biology*. J. M. Polak and S. Van Noorden, editors. Wright-PSG, Bristol, 43-52.
- De Mey, J., M. Moeremans, G. Geuens, R. Nuydens, and M. De Brabander. 1981. High resolution light and electron microscopic localization of tubulin with the IGS (Immuno Gold Staining) method. *Cell Biol. Int. Rep.* 5:889-899.
- Drenckhahn, D., U. Groeschel-Stewart, J. Kendrick-Jones, and J. M. Scholey. 1983. Antibody to thymus myosin: its immunological characterization and use for immunocytochemical localization of myosin in vertebrate non-muscle cells. *J. Cell Biol.* 30:100-111.
- Feramisco, J. R., and S. H. Blose. 1980. Distribution of fluorescently labeled α -actinin in living and fixed fibroblasts. *J. Cell Biol.* 86:608-615.
- Fujiwara, K., and T. D. Pollard. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. *J. Cell Biol.* 71:848-875.
- Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell* 18:193-205.
- Goldberg, B., and H. Green. 1964. An analysis of collagen secretion by established mouse fibroblast lines. *J. Cell Biol.* 22:227-258.
- Goldman, R. D., B. Chojnacki, and M.-J. Yerna. 1979. Ultrastructure of microfilament bundles in baby hamster kidney (BHK-21) cells. The use of Tannic acid. *J. Cell Biol.* 80:759-766.
- Goldman, R. D., E. Lazarides, R. Pollack, and K. Weber. 1975. The distribution of actin in non muscle cells. *Exp. Cell Res.* 90:333-344.
- Goldman, R. D., T. D. Pollard, and J. Rosenbaum, editors. 1976. *In Cell Motility*. Cold Spring Harbor Conference on Cell Proliferation 3 (Books A, B and C). Cold Spring Harbor Laboratories, Cold Spring Harbor, NY. 1373 pp.
- Goldman, R. D., M.-J. Yerna, and J. A. Schloss. 1976. Localization and organization of microfilaments and related proteins in normal and virus-transformed cell. *J. Supramol. Struct.* 5:155-183.
- Gomer, R. H., and E. Lazarides. 1981. The synthesis and deployment of filamin in chicken skeletal muscle. *Cell* 23:529-532.
- Gomer, R. H., and E. Lazarides. 1983. Highly homologous filamin polypeptides have different distributions in avian slow and fast muscle fibers. *J. Cell Biol.* 97:818-823.
- Gordon, W. E. 1978. Immunofluorescent and ultrastructural studies of "sarcomeric" units in stress fibers of cultured non-muscle cells. *Cell Res.* 117:253-260.
- Gordon, W. E., and A. Bushnell. 1979. Immunofluorescent and ultrastructural studies of polygonal microfilament networks in respreading non-muscle cells. *Exp. Cell Res.* 120:335-348.
- Groeschel-Stewart, U. 1980. Immunocytochemistry of cytoplasmic contractile proteins. *Int. Rev. Cytol.* 65:193-254.
- Hatano, S., H. Ishikawa, and H. Sato, editors. 1979. *In Cell Motility: Molecules and Organization*. University of Tokyo Press, Tokyo. 251-261, 417-444, and 445-466.
- Herman, I. M., and T. D. Pollard. 1981. Electron microscopic localization of cytoplasmic myosin with ferritin-labeled antibodies. *J. Cell Biol.* 88:346-351.
- Heuser, J. E., and M. W. Kirschner. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.* 86:212-234.
- Höglund, A.-S., R. Karlson, E. Arro, B.-E. Fredriksson, and U. Lindberg. 1980. Visualization of the peripheral wave of microfilaments in glia cells. *J. Muscle Res. Cell Motil.* 1:127-146.
- Ireland, G. W., and F. C. T. Voon. 1981. Polygonal networks in living chick embryonic cells. *J. Cell Sci.* 52:55-69.
- Jockusch, B. M., and G. Isenberg. 1981. Interaction of α -actinin and vinculin with actin: Opposite effects on filament network formation. *Proc. Natl. Acad. Sci. USA* 78:3005-3009.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Langanger, G., J. De Mey, and H. Adam. 1983. 1,4-Diazobicyclo-[2.2.2]-octane (DABCO) is retarding fading of immunofluorescence preparations. *Mikroskopie* 40:237-241.
- Langanger, G., J. De Mey, M. Moeremans, J. V. Small, and M. De Brabander. 1983. Ultrastructural immunogold marking of actin, α -actinin and filamin in cultured epitheloid and fibroblast cells. *J. Cell Biol.* 97:293a. (Abstr.)
- Langanger, G., J. De Mey, M. Moeremans, J. V. Small, and M. De Brabander. 1984. Immunoelectron microscopic localization of actin, α -actinin and filamin with the IGS (immuno gold staining) method in chicken embryo lung epithelial cells. *J. Submicrosc. Cytol.* 16:43-45.
- Lazarides, E. 1975. Tropomyosin antibody: the specific localization of tropomyosin in nonmuscle cells. *J. Cell Biol.* 65:549-561.
- Lazarides, E. 1976. Actin, α -actinin, and tropomyosin interaction in the structural organization of actin filaments in nonmuscle cells. *J. Cell Biol.* 68:202-219.
- Lazarides, E., and K. Burridge. 1975. α -Actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell* 6:289-298.
- Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307-310.
- Pepe, F. 1966. Some aspects of the structural organization of the myofibril as revealed by antibody staining methods. *J. Cell Biol.* 28:505.
- Rathke, P. C., M. Osborn, and K. Weber. 1979. Immunological and ultrastructural characterization of microfilament bundles: polygonal nets and stress fibers in an established cell line. *Eur. J. Cell Biol.* 19:40-48.
- Sanger, J. M., and J. W. Sanger. 1980. Binding and polarity of actin filaments in interphase and cleaving cells. *J. Cell Biol.* 86:568-575.
- Sanger, J. W., J. M. Sanger, and B. M. Jockusch. 1983. Differences in the stress fibers between fibroblasts and epithelial cells. *J. Cell Biol.* 96:961-969.
- Schollmeyer, J. E., L. T. Furcht, D. E. Goll, R. M. Robson, and M. H. Stromer. 1976. Localization of contractile proteins in smooth muscle cells and in normal and transformed fibroblasts. In *In Cell Motility*. R. D. Goldman, T. D. Pollard, and J. R. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 361-388.
- Small, J. V. 1981. Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshworks. *J. Cell Biol.* 91:695-705.
- Small, J. V., and J. E. Celis. 1978. Filament arrangements in negatively stained cultured cells: the organization of actin. *Eur. J. Cell Biol.* 16:308-325.
- Small, J. V., and A. Sobieszek. 1982. Contractile and structural proteins of smooth muscle. In *Biochemistry of Smooth Muscle*, Vol. 1. N. L. Stephens, editor. CRC Press, Inc., Boca Raton, FL. 85-140.
- Spooner, B. S., K. M. Yamada, and N. K. Wessels. 1971. Microfilaments and cell

- locomotion. *J. Cell Biol.* 49:595-613.
49. Stosel, T. P., J. H. Hartwig, H. L. Yin, K. S. Zaner, and O. I. Stendahl. 1981. Actin gelation and the structure of cortical cytoplasm. *Cold Spring Harbor Symp. Quant. Biol.* 46:569-578.
 50. Strzelecka-Golaszewska, H., E. Prochniewicz, E. Nowak, S. Zmorzynski, and W. Drabikowski. 1980. Chicken-gizzard actin: Polymerization and stability. *Eur. J. Biochem.* 104:41-52.
 51. Taylor, A. C. 1966. Microtubules in the microspikes and cortical cytoplasm of isolated cells. *J. Cell. Biol.* 28:155-168.
 52. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
 53. Wallach, D., P. Davies, and I. Pastan. 1978. Purification of mammalian filamin: similarity to high molecular weight actin-binding protein in macrophages, platelets, fibroblasts and other tissues. *J. Biol. Chem.* 253:3325-3328.
 54. Wang, K., J. F. Ash, and S. J. Singer. 1975. Filamin, a new high-molecular-weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. USA.* 72:4483-4486.
 55. Wang, K., and S. J. Singer. 1977. Interaction of filamin with F-actin in solution. *Proc. Natl. Acad. Sci. USA.* 74:2021-2025.
 56. Weber, K., and U. Groeschel-Stewart. 1974. Antibody to myosin: the specific visualization of myosin-containing filaments in non-muscle cells. *Proc. Natl. Acad. Sci. USA.* 71:4561-4564.
 57. Weber, K., P. Rathke, and M. Osborn. 1978. Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA.* 75:1820-1824.
 58. Webster, R. E., D. Henderson, M. Osborn, and K. Weber. 1978. Three-dimensional electron microscopical visualization of the cytoskeleton of animal cells: immunoferritin identification of actin- and tubulin-containing structures. *Proc. Natl. Acad. Sci. USA.* 75:5511-5515.
 59. Wehland, J., and K. Weber. 1980. Distribution of fluorescently labeled actin and tropomyosin after microinjection in living tissue culture cells as observed with TV image intensification. *Exp. Cell Res.* 127:397-408.
 60. Wehling, R. R. 1983. Purification of HeLa cell high molecular weight actin binding protein and its identification in HeLa cell plasma membrane ghosts and intact HeLa cells. *Biochemistry.* 22:1839-1847.
 61. Wessells, N. K., B. S. Spooner, and M. A. Luduena. 1973. Surface movements, microfilaments and cell locomotion. *Ciba Found. Symp.* 14 (NS):53-77.
 62. Willingham, M. C., S. S. Yamada, P. J. Bechtel, A. V. Rutherford, and I. Pastan. 1981. Ultrastructural immunocytochemical localization of myosin in cultured fibroblastic cells. *J. Histochem. Cytochem.* 29:1289-1301.
 63. Willingham, M. C., S. S. Yamada, P. J. A. Davies, A. V. Rutherford, M. G. Gallo, and I. Pastan. 1981. Intracellular localization of actin in cultured fibroblasts by electron microscopic immunocytochemistry. *J. Histochem. Cytochem.* 29:17-37.
 64. Wulf, E., A. Deboben, F. A. Bautz, H. Faulstich, and T. Wieland. 1979. Fluorescent phalloxin, a tool for the visualization of cellular actin. *Proc. Natl. Acad. Sci. USA.* 76:4498-4502.
 65. Yerna, M. -J., M. O. Aksoy, D. J. Hartshorne, and R. D. Goldman. 1978. BHK-21 myosin: isolation, biochemical characterization and intracellular localization. *J. Cell Sci.* 31:411-429.
 66. Zigmond, S. H., J. J. Otto, and J. Bryan. 1979. Organization of myosin in a submembranous sheath in well-spread human fibroblasts. *Exp. Cell Res.* 119:205-219.